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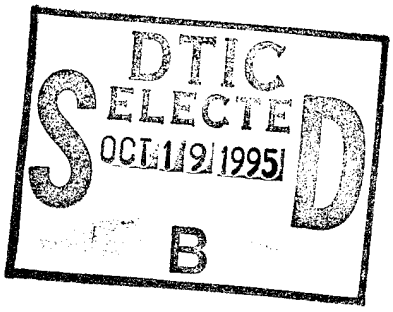

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13. ABSTRACT (Maximum 200 words)

We have produced substantial results related to the objectives of our Department of Defense funding: 1. Identified five *Brucella* proteins and sequenced their respective genes (*L7/L12*, *L10*, *uvrA*, *SSB*, and a transporter containing an ATP binding domain) employing a rapid and direct technique to isolate *Brucella* genes from a genomic library using lymphocytes as probes and obtained two additional genes (*GroEL* and *GroES*) whose proteins also stimulate lymphocyte proliferation. 2. Our recently published data indicates we have successfully identified, isolated, cloned, and sequenced genes and expressed proteins that induce proliferation of lymphocytes from many *Brucella* immunized animals suggesting immunodominant proteins. 3. We now have unprecedented data establishing $CD8^+$ T cells as essential to protection against *B. abortus* infection using MHC class II gene knockout mice. These knockout mice are profoundly deficient in $CD4^+$ T cells but rapidly clear the infection, revealing the pivotal role of $CD8^+$ T cells in protection. Our murine experiments provide the necessary foundation to explore the mechanisms of *Brucella* pathogenesis and *in vivo* protection attributable to *Brucella* proteins that induce T cell responses *in vitro*. We are positioned to apply this practical information to vaccine studies for protection against *B. melitensis* challenge and provide critical data necessary for a human vaccine.

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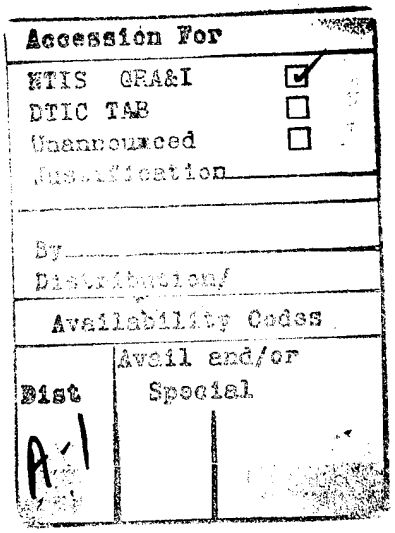

Gary Schiller May 15, 1995
PI - Signature Date

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Introduction

1. The Problem

Brucella melitensis is a gram-negative facultative intracellular bacterium that induces chronic infectious disease by direct contact or by consumption of animal products. The intracytoplasmic localization likely accounts for the great chronicity of the disease; many patients remain ill for months to years. Although the genus *Brucella* consists of six species that exhibit preferential host adaptation (1), greater than 98% homology of various *Brucella* species occurs at the DNA level (2). Therefore, proteins encoded by the DNA must be very similar, if not identical. In fact *Brucella melitensis*, *B. suis* and *B. abortus* can all infect humans with similar serious disease consequences.

Natural infections occur when the organisms penetrate the mucosa of the nasal, oral or pharyngeal cavities. Following penetration, the bacteria are transported either free or within phagocytic cells to regional lymph nodes where hyperplasia and inflammation occur. Secondary localization in other lymph nodes, spleen, liver, and bone where granulomas form. Viable bacilli may persist for several months in the granulomas, giving rise to acute or chronic symptoms. For this reason the incubation period in brucellosis may be very long.

Two species, *B. abortus* and *B. melitensis* have particular relevance to humans. *B. abortus* primarily affects cattle causing abortion or the birth of severely debilitated calves and contaminated dairy products serve as a source for human infection. *B. melitensis* is frequently the cause of human brucellosis in countries other than the United States and is found particularly in the Mediterranean region. In Israel, bovine brucellosis and human brucellosis is increasing (3, Israel Veterinary Services, personal communication, 1995). In 1984, 1985 and 1987 the incidence was 193, 225 and 280 human cases, respectively. The incidence of human brucellosis in the West Bank was 30 and 36 cases per 100,000 inhabitants in 1985 and 1986, respectively. All bacteriologically confirmed human Brucellosis cases have resulted from *B. melitensis* infection. Goat and sheep herds are infected with *B. melitensis* and unpasteurized commercial milk products are a frequent source of this human infection. Therefore, both rural and urban populations are affected. The total number of small ruminant animals has increased significantly in recent years and is currently estimated at 350,000 animals. Because most of the animals are kept in small family herds that occupy common grazing territories, the opportunity for infection is high.

Of particular concern to the military, *B. melitensis* has potential use as a biologic warfare agent against U.S. troops. Presently, a human vaccine does not exist. Thus, there is an immediate need to develop a functional, practical vaccine that affords protection in the field to military personnel.

2. Background of Previous Work

Since the early experiments of Mackaness with *Listeria* (4) and others with *B. abortus* (5), cellular immunity is assumed to have an important role in brucellosis. Macrophages serve as key cells in maintaining the facultative intracellular bacterium and in presenting *Brucella* antigens to T lymphocytes for the initiation of an immune response.

Several lines of evidence suggest that cellular immunity limits the pathogenesis of brucellosis. The preferential migration of *Brucella*-specific bovine lymphocytes from a primary

challenged lymph node to a secondarily challenged site confirms the ability of T lymphocytes specific for *Brucella* epitopes to participate in the in vivo immune response (6). Macrophages serve as important resident cells for *B. abortus*, and these cells phagocytize and kill most ingested species of bacteria within a few hours (7, 8). Resistance depends on successful interaction between T lymphocytes specific for particular bacterial antigens and macrophages (9). *Brucella* proteins that induce a T lymphocyte mediated response are most likely responsible for the observed protection.

Vaccination of cattle with *B. abortus* strain 19 usually results in a transient rise in antibodies specific for the bacterial lipopolysaccharide (LPS). Passive transfer of anti-LPS antibody alone is not protective for cattle, although some protection is afforded in mice (10). Greater protection to *B. abortus* has been shown in mice by adoptive transfer of $CD4^+$ and $CD8^+$ lymphocytes (10, 11), but similar adoptive lymphocyte transfer experiments have not been possible in cattle or humans. Also, recent information (12) as well as our own unpublished work suggests in the mouse that $CD8^+$ lymphocytes are more important than $CD4^+$ lymphocytes in the resolution of brucellosis infection.

Therefore, determining the lymphocyte populations induced following immunization and the ability of these immune cells to protect animals against bacterial challenge are important steps in testing the hypotheses underlying vaccination.

3. Purpose of the Present Work

Our overall aim was to identify the immunodominant *Brucella* proteins important for T lymphocyte responses. The specific objectives were to:

a. Identify and define by DNA sequence analysis *Brucella melitensis* antigens recognized by T lymphocytes.

b. Express the recombinant bacterial proteins and verify that T lymphocytes from mice recognize the recombinant protein.

Body of Current Progress

a. Identify and define by DNA sequence analysis *Brucella melitensis* antigens recognized by T lymphocytes

We have made rapid progress in the identification of *Brucella* proteins that stimulate lymphocyte proliferation (13-17). Individual *Brucella* proteins that induced lymphocyte proliferation were characterized by one- and two-dimensional cellular immunoblotting, and thirty-eight *Brucella* proteins that induced lymphocyte proliferation were resolved (13). In addition, lymphocytes from *B. abortus* vaccinated animals proliferated in response to proteins isolated from other *Brucella* species (14). These results indicate that a genus-wide subunit vaccine may be feasible.

Of particular interest, two immunogenic proteins, 12 and 31 kDa were subjected to partial N-terminal amino acid analysis. An oligonucleotide probe was constructed that successfully identified the *L7/L12* gene (GenBank #L23505) and associated *L10* gene (GenBank #L19101) that comprise the rplJL operon (17). These genes encode the L10 and L7/L12 proteins, essential for bacterial ribosomal function and protein synthesis. We then confirmed that the L7/L12 protein represents the immunodominant 12 kDa protein previously identified on immunoblotting (18).

We have extended these findings by cloning and sequencing additional *Brucella* genes encoding proteins that stimulate lymphocytes from immunized animals. Using *Brucella* proteins

expressed from a pBluescript II SK$^-$ genomic library **Fig. 1**, we have isolated additional genes encoding antigens that induce proliferation by lymphocytes primed to the bacterium (16). The *B. abortus uvrA* gene (GenBank #L10843) encodes a protein that stimulated T lymphocyte proliferation in all three animals tested, suggesting that it may represent another immunodominant antigen. The uvrA protein is responsible for repair of *Brucella* DNA. The *SSB* gene that genomically overlaps the *uvrA* gene was also identified. The SSB protein is responsible for binding single stranded *Brucella* DNA and does not stimulate T cell proliferation. Another protein, a transporter containing an ATP binding domain that stimulates T cell proliferation from several animals, has been identified from screening our *Brucella* genomic library using T lymphocytes. The gene encoding this protein has been cloned and sequenced, and the gene contains Walker motifs for ATP binding. This gene appears to belong to the hemolysin B family of proteins found in *Escherichia coli* and likely encodes a transporter protein that transports the toxin outside the bacterium. In *E. coli* the hemolysin B protein is a serious human toxin. We have not yet found a hemolysin toxin in Brucella. *Brucella* mutants lacking this gene are planned to determine the functional importance of this protein. We have also obtained from other workers two additional *Brucella* genes, *GroES* and *GroEL*, whose encoded proteins are heat shock proteins. We have expressed these genes and the proteins stimulate T lymphocyte proliferation.

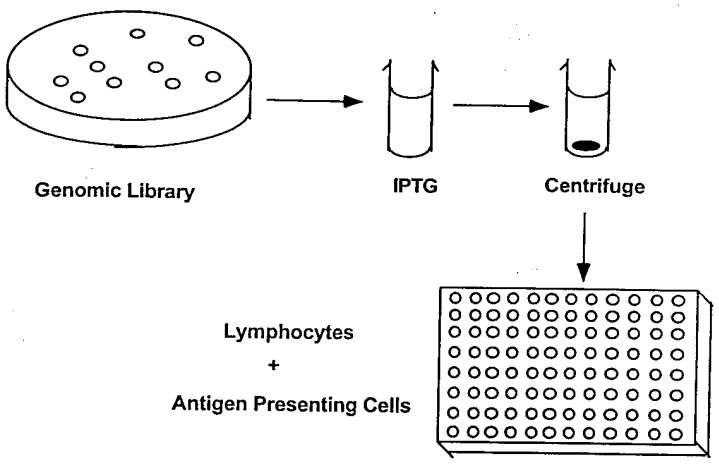

Fig. 1. Screening a Brucella genomic library for bacterial proteins inducing T lymphocyt proliferation. Individual colonies were selected and cultured overnight in the presence of IPTG followed by centrifugation. The resulting supernatant was cultured with antigen presenting cell and lymphocytes from Brucella primed animals.

Presently we have 7 *Brucella* genes. Five of these genes encode proteins that stimulate T lymphocyte proliferation from a number of animals. Immunodominant proteins are defined as antigens to which the immune response of the majority of animals is directed. Therefore, determining the protective potential of these proteins would be a first step in testing a hypothesis relating *in vitro* T cell recognition and *in vivo* protection. Lymphocytes that respond to proteins expressed from single bacterial clones provide a novel strategy to identify an array of candidates for potential protective vaccines. Our proven methodology using *Brucella* primed T lymphocytes has yielded a collection of proteins that uniquely position us to develop a defined protective vaccine.

b. Express the recombinant bacterial proteins and verify that T lymphocytes from mice recognize the recombinant protein.

The *Brucella* L7/L12 ribosomal gene as well as four addition *Brucella* genes have been amplified by PCR and subcloned into the prokaryotic expression vector pMAL-c2 to produce a fusion protein consisting of the maltose binding protein and the *Brucella* protein of interest. *E. coli* DH5α was transformed with the pMAL-L7/L12 construct, and gene expression was induced by isopropyl-β-thiogalactopyranoside (IPTG).

Expression of a 55-kDa fusion protein was observed by SDS-PAGE containing lysates from IPTG-induced *E. coli* that contained the pMAL-L7/L12 construct (**Fig. 2A**). To confirm that the expressed fusion protein was MBP-L7/L12, Western blot analysis was performed, using rabbit anti-MBP antibody. **Fig. 2B** shows the anti-MBP recognition of the MBP-L7/L12 fusion protein. After expression, the fusion protein was purified in an amylose affinity column, and the interval necessary for cleavage of L7/L12 from MBP was determined by kinetic experiments at 2,4,8, 14 hours with factor Xa treatment. Incubation of factor Xa protease with the fusion protein for 14 hours resulted in total cleavage of MBP from L7/L12 (**Fig. 3**). Cleavage of the fusion protein shown in **Fig. 3** confirmed the expected molecular masses of MBP and L7/L12, 42.7 and 12 kDa, respectively. Recombinant L7/L12 was purified by rebinding MBP to the amylose resin. **Fig. 4A** shows the isolated recombinant L7/L12 on an SDS-PAGE after purification. Western blot analysis demonstrated that the anti-MBP antibody did not recognize the separated recombinant L7/L12 (**Fig. 4B**).

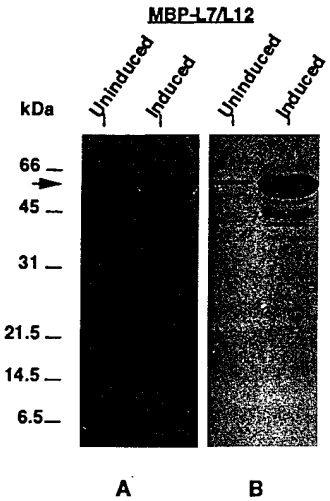

FIG. 2 SDS-PAGE profile and corresponding Western blot analysis of the recombinant MBP-L7/L12 fusion protein. (A) Coomassie blue-stained SDS–15% PAGE of lysates from uninduced and induced *E. coli* expressing the pMAL-L7/L12 construct. (B) Immunoblot of the recombinant MBP-L7/L12 fusion protein probed with rabbit anti-MBP antibody.

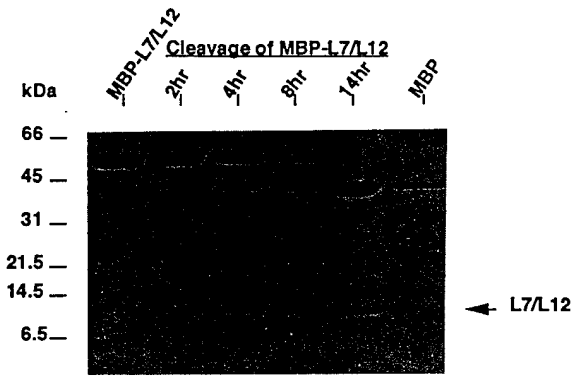

FIG. 3. SDS-PAGE analysis of MBP-L7/L12 fusion protein cleavage by treatment with factor Xa protease at 2, 4, 8, and 14 h. Pure MBP and noncleaved MBP-L7/L12 were used as controls. Molecular mass markers are shown on the left, and pure L7/L12, released from MBP, is indicated by the arrow on the right.

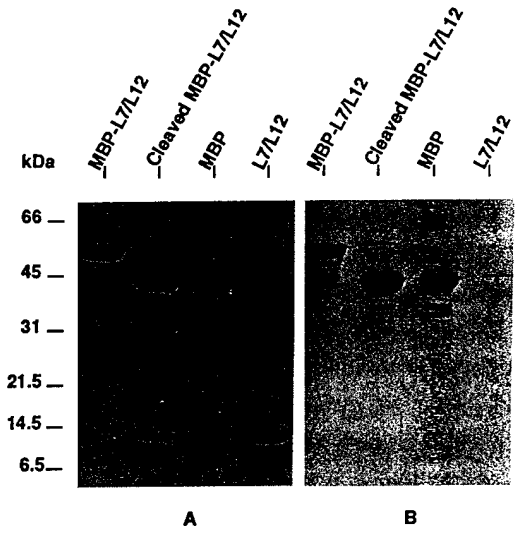

FIG. 4 (A) Coomassie blue-stained SDS–15% PAGE profile of MBP-L7/L12 fusion protein, cleaved MBP-L7/L12, pure MBP, and purified L7/L12 ribosomal protein. (B) Electrotransferred samples from SDS-PAGE were analyzed by immunoblotting with rabbit anti-MBP antibody.

Primed lymphocytes from three different animals proliferated to the recombinant L7/L12 protein, whereas cells from naive animals did not (**Fig. 5**). Using monoclonal antibodies to cell surface markers, the phenotype of the responding cells were determined using flow cytometry. At day seven the proliferating cells were $CD4^+$ (20.7% ±0.8%), $CD8^+$ (13.2% ± 1.6%), IgM (10.7% ± 2.1%), and γδ-T cell receptor (4.5% ± 1.0%). We detected a twofold increase in the percentage of $CD4^+$ T lymphocytes when cells were stimulated in vitro with the recombinant L7/L12 (20.7% ± 0.8%) compared to cells cultured in medium alone (10.8% ± 1.5%). However, no significant change in the percentage of the other cell types was detected.

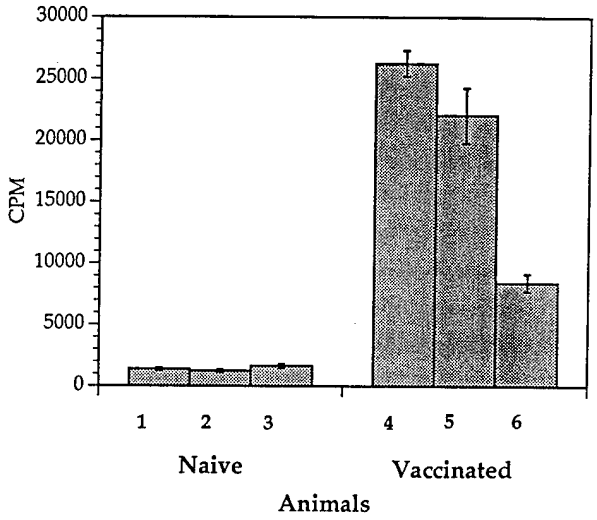

FIG. 5. Proliferative response of PBM cells from *B. abortus*-vaccinated cattle and naive animals. PBM cells were stimulated with 50 μg of rL7/L12 or concanavalin A per ml. Concanavalin A-stimulated cells from both groups of animals had responses of >100,000 cpm (data not shown). Results are expressed as mean cpm. Error bars indicate standard errors of the means.

Ribosomal preparations from several pathogens, including *Brucella spp.* have been shown to be highly protective vaccines (19-22). However, the components of these preparations have not yet been characterized. A Salmonella typhimurium ribosomal vaccine afforded cell-mediated protection, based on the capacity of primed T cells to respond to *Salmonella* antigens and to activate macrophages (20). The mechanism by which the host responds to and eliminates infection is a central issue in our understanding of the immune response to pathogens. For many intracellular organisms, such as *Brucella spp.*, T cell mediated immunity is critical for host protection. Because T cells play a major role in protection against *Brucella* infection through cytokine secretion, such as that of interferon-γ (IFN-γ), the identification of specific *Brucella* antigens that induce a T lymphocyte response is an important element in designing new molecular candidate vaccines.

The importance of $CD4^+$ and $CD8^+$ lymphocytes in the resolution of murine brucellosis has been implicated in several studies (10-12). Immunity to *B. abortus* crucially depends on antigen-specific T cell mediated activation of macrophages for killing this organism. We have examined the pattern of T-helper cell response from infected BALB/c mice after *in vitro* stimulation with recombinant L7/12 ribosomal protein or γ-irradiated *B. abortus*. In addition to antigen-specific proliferation, $CD4^+$ T cells were tested for interleukin-2 (IL-2), IL-4 and IFN-γ mRNA expression and secretion. Detection of cytokine transcripts and secreted cytokines was performed using reverse transcriptase (RT)-polymerase chain reaction (PCR) and specific ELISA assays. Primed $CD4^+$ T cells proliferated to the recombinant protein or whole *B. abortus*. The functional cytokine profile of the proliferating cells was typical of a Th1 cell phenotype, as we detected transcripts for IL-2 and IFN-γ but not IL-4 (18). Among the cytokines analyzed, only IFN-γ produced in the Th cell culture supernatants was detected by ELISA following either bacterial or recombinant protein stimulation of the lymphocytes. Thus, L7/L12 ribosomal protein and γ-irradiated *B. abortus* preferentially stimulated IFN-γ-producing Th1 cells after *in vitro* stimulation. The results of this study provide for the first time an explanation of why ribosomal vaccines may protect against intracellular infections, and an experimental basis for identifying polypeptides from a pathogen that stimulates the desired cytokine profile and Th cell response crucial for the design of genetically engineered candidate vaccines. We are now poised to test lymphocyte cytokine profiles in mice immunized with the L7/L12 gene in combination with additional *Brucella* genes.

Necessary for vaccine design is an understanding of the immune cells required for protection. We have determined the phenotype of lymphocytes critical for host resistance to *B. melitensis*. Particularly in the case of murine brucellosis, earlier studies reported that either $CD4^+$ (10, 23-25) or $CD8^+$ T cells (12) or both (10) are required for protection in *Brucella* infected mice. Recent studies evoked a central role for $CD8^+$ T cells in protection against intracellular pathogens (26, 27). We used genetically engineered "knockout" mice to assess the role of T cell subsets in *Brucella* infection. MHC class I knockout mice carry a disrupted gene for B2-microglobulin (β2-m) and lack functional $CD8^+$ T cells. MHC class II "knockout" mice carry a defective gene for the H2-I-Aβ chain, and hence the MHC class II Aβ heterodimer is nonfuctional. MHC class II knockout mice are severely deficient in CD4 T helper cells. C57BL/6 mice served as a control group because this strain has been shown to be inherently "resistant" to brucellosis and provided a comparison of the C57BL/6 T cell response to our existing data from BALB/c mice.

Murine brucellosis was markedly exacerbated in MHC class I knockout mice compared to

class II knockout or C57BL/6 mice **Fig. 6**, strongly implicating $CD8^+$ T cells in acquired resistance to brucellosis. Splenocytes from *Brucella* primed knockout mice and C57BL/6 mice exhibited a Th type I cytokine profile marked by elevated IFN-γ mRNA expression and cytokine secretion as evidenced by RT-PCR and ELISA assays (**Fig. 7**). Only basal levels of IL-2 and IL-4 transcripts were detected. Our cytokine profile investigations were expanded to include TGF-β and IL-10 with interesting results. *Brucella* did not induce secretion of TGF-β but substantial IL-10 transcription and secretion was detected in spleen cell supernatants from both knockout and control mice. Interestingly, MHC class I knockout mice, unable to clear infection, produced twofold higher amounts of IL-10 than either class II knockout mice or the control group. These data correlate with reports by others that *Mycobacterium avium* induces production of IL-10. IL-10 is best understood as a cytokine synthesis inhibitory factor (28, 29); however, IL-10 has been shown to inhibit monocyte MHC class II expression as well as intracellular killing of parasites (30, 31). Thus, vigorous transcription of IL-10 in mice unable to rapidly clear *Brucella* infection strengthens our findings that $CD8^+$ T cells are central to a successful immune response.

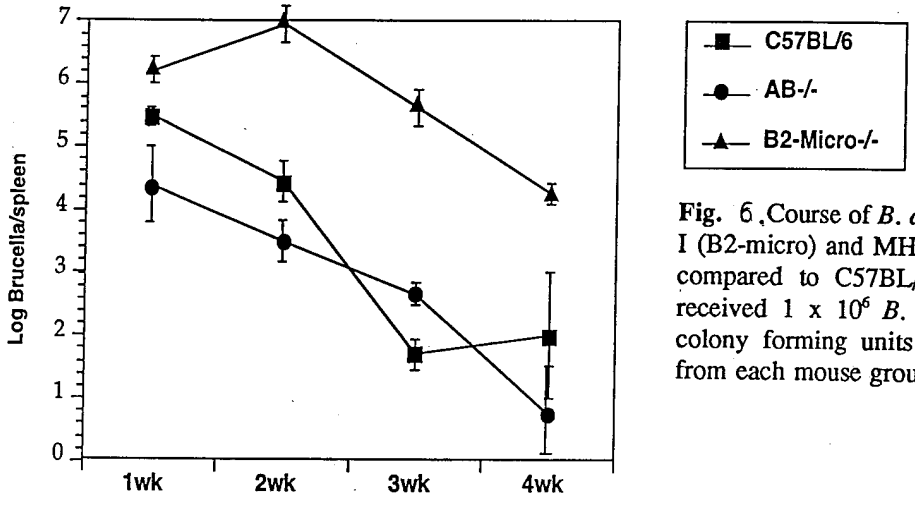

Fig. 6, Course of *B. abortus* infection in MHC class I (B2-micro) and MHC class II (Aβ) deficient mice compared to C57BL/6 resistant mice. All mice received 1 x 10^6 *B. abortus* S19 and the splenic colony forming units of bacteria were determined from each mouse group for four weeks.

A purified population of $CD8^+$ T cells from MHC class II knockout mice and C57BL/6 mice displayed a $CD44^{hi}$ $CD45RB^{low}$ phenotype as well as a Th1 type cytokine transcription profile featuring high levels of IFN-γ mRNA. This, again, is quite interesting in that IL-10 has been shown to inhibit IFN-γ in Th1 $CD4^+$ T cells (28, 29, 32). Additionally, we have shown that $CD8^+$ cytotoxic T cells from C57BL/6 mice potently kill Brucella-infected macrophage target cells. Taken together, our data powerfully suggests that $CD8^+$ T cells merit more focused investigation.

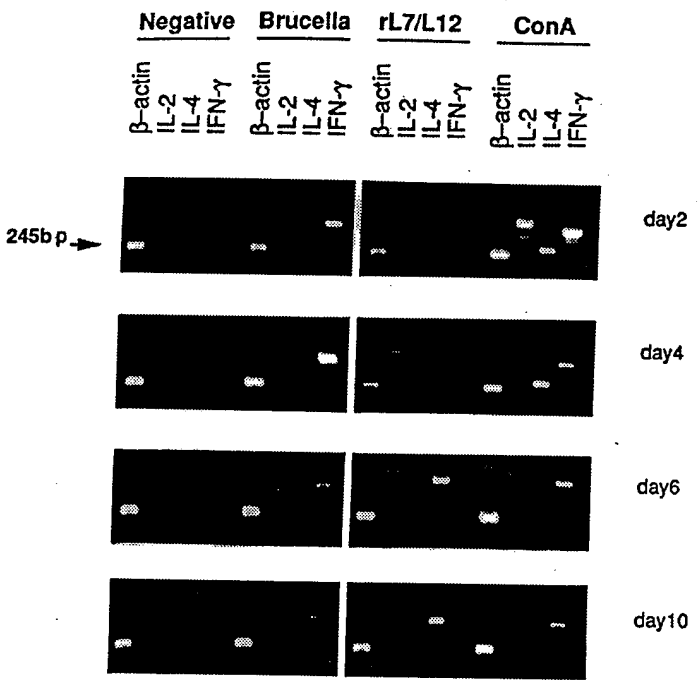

Fig. 7. Pattern of β-actin, IL-2, IL-4, and IFNγ mRNA expression of CD4⁺ T cells *from B. abortus* infected mice. The cells were cultured with L7/L12, irradiated *B. abortus* or Con A. At day 2, 4, 6 and 10 of culture total RNA was extracted, and the mRNA expressed was determined by RT-PCR. Transcripts from CD4⁺ T cells cultured with medium alone were analyzed as a negative control.

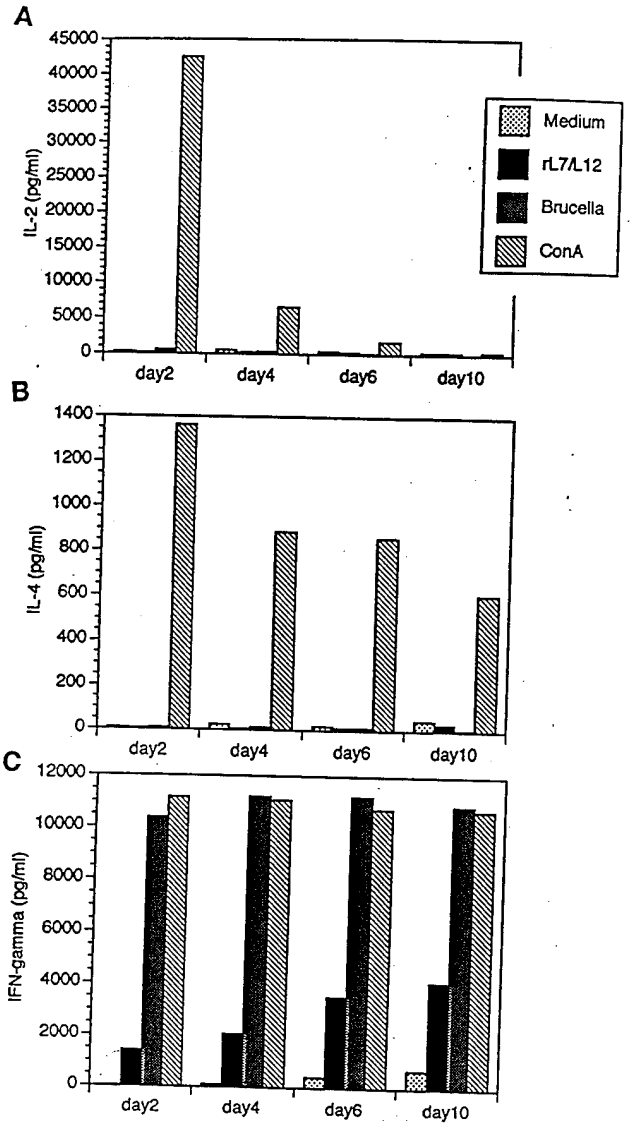

Fig. 8. Cytokine responses of CD4⁺ T cells from *B. abortus* infected BALB/c mice and stimulated in vitro with L7/L12, irradiated *B. abortus* or Con A. ELISA assays for IL-2 (A), IL-4 (B), and IFNγ (C) present in cell culture supernatants at day 2, 4, 6 and 10 are shown. Supernatants of cells cultured with medium alone were tested as a negative control.

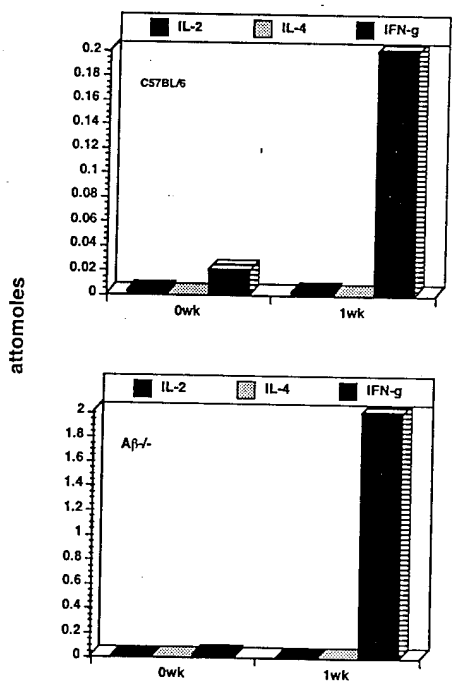

Fig. 9. Cytokine gene expression in *B. abortus* primed CD8⁺ T cells using a competitive RT-PCR based assay. Spleen cells were separated by cell sorting to 99% CD8⁺ T cells and mRNA obtained. The RT-PCR products were quantitated in comparison with competitive internal standards for IL-2, IL-4 and IFNγ using a gel video system (NIH Image 1.54) to scan and quantitate ethidium bromide stained gel bands.

Conclusions

In summary, we have produced substantial results related to the objectives of our Department of Defense funding: 1. Identified five *Brucella* proteins and sequenced their respective genes (*L7/L12*, *L10*, *uvrA*, *SSB*, and a transporter containing an ATP binding domain) employing a rapid and direct technique to isolate *Brucella* genes from a genomic library using lymphocytes as probes (16, 17) and obtained two additional genes (*GroEL* and *GroES*) whose proteins also stimulate lymphocyte proliferation. 2. Our recently published data indicates we have successfully identified, isolated, cloned, and sequenced genes and expressed proteins that induce proliferation of lymphocytes from many *Brucella* immunized animals suggesting immunodominant proteins (13-16, 18). 3. We now have unprecedented data that establishes $CD8^+$ T cells as essential to protection against *B. abortus* infection using MHC class II gene knockout mice. These knockout mice are profoundly deficient in $CD4^+$ T cells but rapidly clear the infection, revealing the pivotal role of $CD8^+$ T cells in protection. Our murine experiments provide the necessary foundation to explore the mechanisms of *in vivo* protection attributable to *Brucella* proteins that induce T cell responses *in vitro*. Characterizing the *in vivo* role of these individual *Brucella* proteins in protection will simultaneously better our understanding of *Brucella* pathogenesis and provide critical data necessary for a human vaccine. We are now positioned to apply this practical information to vaccine studies for protection against *B. melitensis* challenge.

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